Self-organization of stem cells into embryos: a window on early mammalian development

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Embryonic development is orchestrated by robust and complex regulatory mechanisms acting at different scales of organization. *In vivo* studies are particularly challenging for mammals after implantation, due to the small size and inaccessibility of the embryo. The generation of stem cell models of the embryo represents a powerful system to dissect this complexity. Control of geometry, modulation of the physical environment, and priming with chemical signals reveals the intrinsic capacity of embryonic stem cells to make patterns. Adding the stem cells for the extraembryonic lineages generates three-dimensional models that are more autonomous from the environment and recapitulate many features of pre- and post-implantation mouse embryo, including gastrulation. Here, we review the principles of self-organization and how they set cells in motion to create an embryo.

Introduction

Vertebrate development deploys orthologous sets of genes that first create the body axes: anterior-posterior (AP), dorsal-ventral (DV) and left-right (LR), as well as the germ layers: endoderm, mesoderm and ectoderm, and ultimately refines these patterns to the diverse adult forms we know. How are these spectacular feats of self-organization possible?

Although we have an inventory of genes that confer cell identity, we are far from understanding how their products communicate to generate embryonic patterns. Multiple levels of regulation add robustness to embryonic development but this redundancy makes the regulatory network difficult to decipher. Using stem cells as a model system to study embryology, we are now able to start peeling back these layers of regulation to reveal the dynamic organization of the embryo. Technical advances that created stem cell embryology were reviewed in (*1*). Here we focus on the principles of how cell communication at the molecular level enables embryonic self-organization in mouse and human embryos.

Mammalian embryo development

Pre-implantation development is fairly conserved among mammalian species (*2*). Fertilization leads to a step-wise process of cell fate specification that culminates with the blastocyst comprising three cell types: the embryonic epiblast and the extra-embryonic primitive endoderm and trophectoderm (*3*-*6*). Blastocyst implantation initiates a dialogue between the uterus and the embryo, which leads to

the reorganization of both the embryo and the maternal tissues. Across diverse mammalian species, the basic relation between tissues is conserved, but post-implantation conceptuses present distinct embryonic architectures, from the cylinder-like shape of mouse embryos to the bilaminar disc of human embryos (*2*) (Fig. 1). How these different shapes evolved remains unknown.

Interactions between embryonic and extra-embryonic tissues are critical to reshape the developing embryo. In mouse, the polar trophectoderm proliferates in response to FGF4 secreted by the epiblast to form the extra-embryonic ectoderm (*7*), which will form the placenta. Concomitantly, the epiblast and extra-embryonic ectoderm undergo a process of lumenogenesis in response to extra-cellular matrix (ECM) secreted by the primitive endoderm-derived visceral endoderm (*8, 9*). The fusion of the extra-embryonic ectoderm and epiblast cavities leads to the formation of the pro-amniotic cavity (*10*), fundamental for the establishment of the body plan. This coincides with a symmetry breaking event to form the anterior signaling center in the visceral endoderm (AVE) that defines the AP axis and the site of gastrulation (*11-13*).

In human embryos the epiblast undergoes lumenogenesis in a similar way to the mouse with one important difference: epiblast in contact with the trophoblast forms the amniotic epithelium whereas epiblast in contact with the hypoblast (visceral endoderm-equivalent) forms the epiblast disc (*1, 2, 14*). The mechanisms of symmetry breaking leading to AP axis formation in human embryos remain

unknown, but mechanical and chemical cues are clearly involved. In Cynomolgus monkey embryos a population of hypoblast cells that expresses Wnt and Nodal inhibitors (DKK1 and CER1), characteristic of the mouse AVE, has been identified (*15*).

Is a dialogue between mother and embryo required for this morphogenesis? Comparative embryology provides a preliminary answer. In mammalian embryos such as pig, rabbit, and cow, embryonic morphogenesis and gastrulation take place before implantation (*16, 17*). Mouse and human embryos can undergo early post-implantation morphogenesis without maternal input (*8, 18-21*). Even if the uterine environment could help to modulate these events (*22, 23*), the self-organizing capabilities of mammalian embryos (and stem cells) are becoming increasingly apparent.

Modes of self-organization

Although a system composed of invariant parts might be induced to *self-assemble*, here we focus mainly on *self-organization* that encompasses both patterning (fate change) by exchange of signals as well as cell rearrangements. To further refine terminology, consider a supersaturated vapor that is spatially homogeneous until droplets nucleate and grow. The immediate trigger for a drop may be a speck of dust but its subsequent expansion is reproducible. This is an example of *spontaneous symmetry breaking,* since the initially homogeneous vapor (the symmetric state)

becomes an inhomogeneous mist of droplets.. Analogous, self-organization occurs in systems of chemical reactions with diffusion where Turing showed inhomogeneities with a characteristic spatial scale result from a random trigger to a uniform but unstable system (*24*). Embryology generally avoids spontaneous symmetry breaking since the outcome is too fragile; rather it proceeds by progressive refinement of prior asymmetries, still suggestive of Turing's ideas.

The requirements for Turing instability are intuitively transparent: an activator induces the production of its own inhibitor but the inhibitor diffuses more rapidly than the activator and confines the activator in space. This has the seemingly paradoxical consequence that the peak expression of both the activator and inhibitor are in the same place, rather than being opposed. Both modes of regulation are seen in the mouse embryo (*25*).

Since signaling pathways often involve secreted inhibitors, Turing phenomena are frequently posited. However, there are many confounding influences as exemplified by studies of digits and feather follicles in the skin (*26-28*). Reaction-diffusion systems can also account for the 'community effect', articulated by John Gurdon, whereby a tissue forces the majority fate on cells within it (*29-31*).

In quantitative analogy to the surface tension driven separation of oil and water, cells of different types can sort by differential adhesion (*32*). Chemotaxis can also

contribute to pattern formation as in sporulation in Dictyostelium, and signaling pathways themselves can provide chemotactic signals (*33*).

Self-organization in embryonic stem cells

The disc shape of the human epiblast suggests the possibility of a 2D model. Embryonic stem cells (ESCs) naturally supply the epiblast. The extra-embryonic hypoblast, and spatial confinement are modeled by micropatterns: slides with arrays of disks where ECM proteins bind and control where cells adhere. The extra-embryonic trophoblast is modelled by addition of BMP4 to the media to provide the morphogen trigger (*34*). As envisioned by Tam (*35*), the cells pattern with concentric rings of endoderm and mesoderm and a central disk of anterior epiblast (Fig. 2 and 3). The mesendoderm cells express the same markers and require the same signals (Wnt and Activin/Nodal induced downstream of BMP4) as does the mouse primitive streak, and the same secreted inhibitors are required to spatially confine the streak and shield the central epiblast from morphogens. Thus a homogeneous layer of human ESCs can self-pattern on a scale of ~2000 cells, without contribution from extra-embryonic lineages. Similarly, micropattern culture guides self-organization of ectodermal derivatives (*36*).

The micropattern system facilitates deciphering how cell fates are defined by distance from the colony boundary (*37-40*). hESCs are apico-basally polarized and the BMP and Activin/Nodal receptors are basolateral and not accessible to apical

ligands except at the colony boundary. The secreted BMP inhibitor NOGGIN also restricts signaling to the colony boundary and is active from the apical side, suggesting complex signal transmission in polarized epithelia (*41*).

Receptor localization in the micropattern system, when folded into a cup-shape as in the mouse, helps explain why the pro-amniotic cavity (facing the apical side of the epiblast) does not short circuit the proximal-distal patterning and why the initial BMP response is proximal only (*41*). This conjecture was confirmed by mistargeting the BMP receptors in the embryo (*42*).

Micropattern culture has been extended to the mouse (*43*). When mouse ESCs are differentiated to a post-implantation-like state (*44*) and transferred to micropatterns, they display properties similar to the pre-gastrulation epiblast. Differentiation with Wnt/Activin and BMP gives fates indicative of distal vs proximal streak derivatives.

Embryonic stem cells have been shown to self-organize in 3D culture. When ESCs are cultured in a 3D gel supplemented with ECM, they form an apical-basal polarized shell that eliminates the boundaries of micropattern culture, allowing control of substrate mechanics and chemistry (*45-49*). Human pluripotent cells cultured in such a system respond to BMP by polarizing and breaking symmetry into anterior epiblast and posterior primitive streak in a Wnt dependent manner (*50*).

A synthesis of signals and mechanics can be achieved by placing hESCs on a 3D soft gel and doping the media above with a low concentration of ECM components (*51, 52*). This treatment induces the patches to fold into closed polarized shells, which depending on the initial cell density can generate squamous, asymmetric, or columnar cysts. Squamous cysts represent an amnion-like tissue based on gene expression, cell shape, and BMP signaling activity (*51*), also active in the amnion of cynomolgous monkeys (*15*). Columnar cysts represent the epiblast, and asymmetric cysts undergo a symmetry-breaking event to form an amnion-like hemisphere and an epithelia-like disc (Fig. 2 and 3). The morphogenesis of the resulting amniotic sac plausibly requires BMP that induces Brachyury expression and EMT in the putative epiblast, but the mechanisms of symmetry breaking remain unknown. With the ability to define a gel surface in 3D, future studies will shed light onto how morphology influences cell-cell signaling.

Embryoid bodies offer an alternative approach for eliciting the self-organizing potential of stem cells (*53, 54*). When a clump of mESCs is given a pulse of Wnt agonist, it elongates into a tube showing markers for AP, DV, and LR axes (*55, 56*) (Fig. 2). These so-called *gastruloids* recapitulate the spatiotemporal patterns of gene expression of embryos after gastrulation, such as HOX genes in the correct temporal order and in nested telescoping domains (*57*).

Self-organization of embryonic and extra-embryonic stem cells

These ESC-only based models are informative in revealing how homogenous populations of cells can give rise to different cellular fates through the process of self-organization. However, these models differ from natural embryos in their lack of extra-embryonic tissues, which are critical for development and provide spatial context for signaling interactions. For this reason, new stem cell embryo models have been developed that incorporate interactions of ESCs with extra-embryonic cells (*58-61*) (Fig. 2 and 3).

Combining ESCs and trophoblast stem cells (TSCs) (Fig. 1) in ECM (*58*) to substitute for the basal membrane produced by the primitive endoderm, leads to the generation of post-implantation embryo-like structures. In this model, cells polarize and form lumens in the ESC-derived embryonic and TSC-derived extra-embryonic compartments that then join, in response to Nodal signaling (*58, 60*). A domain of asymmetric Brachyury expression develops at the boundary between the ESC and TSC compartments. These *polarized embryo-like structures* induce mesoderm formation but do not proceed through gastrulation (Fig. 2 and 3). This event has been observed after substituting the ECM with the third stem cell type, extra-embryonic endoderm (XEN) stem cells (Fig. 1), which provide the natural basement membrane (*59, 60*). As a result the formed structures look remarkably like early post-implantation embryos in morphology, gene expression, and signaling communication. They break symmetry at the embryonic and extra-embryonic *boundary* with the induction of AP patterning and EMT leading to mesoderm and definitive endoderm formation (*59*). This self-organization occurs in response to

BMP and Wnt signaling active during the cavity fusion process (*58, 59*). Finally, the markers of primordial germ cells become expressed in a spatial-temporal manner characteristic of development. These structures induce decidualization upon their transfer to murine foster mothers, but don't develop further.

The self-assembly and subsequent self-organization into so-called *gastrulating embryo-like structures* are possible because the different stem cell types not only establish signaling among themselves, but also provide the building blocks for spatial morphogenesis. The migration of ESCs to form the mesoderm layer, sandwiched between ESC-derived epiblast and XEN-derived visceral endoderm, and replacement of the XEN-layer with definitive endoderm are the hallmarks of early-to-mid gastrulation (*59*). This points to the essential requirement for the correct choreography of cells from embryonic and the two extra-embryonic tissues to achieve correct form.

Will stem cell models ever pass the ultimate test of function which is development following implantation? Combining ESCs and TSCs in a non-adherent platform leads to the generation of pre-implantation embryo-like structures remarkably similar to blastocysts, both in terms of shape, gene expression, and intercellular communication (*61*) (Fig. 2). These so-called *blastoids* can also induce decidualization but then their development stops (Fig. 3). The derivation of extra-embryonic stem cells that better match the expression signatures of real embryos should improve the morphology of these embryo-like structures *(62, 63)*. Similarly,

the recent generation of expanded potential stem cells, which have the ability to form both embryonic and extra-embryonic tissues, represent a promising tool for future research (*64, 65*)

Conclusions and Perspectives

Stem cell models of embryogenesis allow independent control of shape, mechanics, and means to juxtapose embryonic and extra-embryonic tissues. Therefore, they represent powerful systems to address classic questions of embryology. For example, does gastrulation proceed in embryo-like structures of anomalous size, or does size have to be regulated first? Which combination of chemical and mechanical signals suffices to trigger primitive streak formation? To which extent can we induce gastrulation without AVE? Do the genetic barriers to chimerism operate through the same pathways as intra-species cell competition? Stem cell systems will thus illuminate the genetic determinates of size and timing control.

Stem cell-derived embryos are models of development, and therefore they cannot fully recreate all the complexity of developing organisms. The field of stem cell embryology is in its infancy and will expand by tuning chemical and physical parameters, and using stem cell lines with broader developmental potential (*64, 65*). Particularly interesting would be the combination of human ESCs with human TSCs (*66*), and potentially human hypoblast stem cells, to recapitulate the human conceptus.

However, in devising these studies, it is important to consider when stem cell models of embryos acquire the protections attached to human embryos. Is a collection of cells that mimics gastrulation any more human than a brain organoid that might one day be endowed with sensory primordia? (*67*). It is clearly unethical to implant a stem cell-derived embryo into a human, yet many pregnancies fail or are impaired by placentation. Can stem cell models help to address this problem?

The promise for basic science is clear. Building embryos from stem cells, like the *in vitro* reconstitution of biochemical systems from purified components, is the test of whether we can understand the whole from the parts.

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Figure legends:

Figure 1: Schematic representation of mouse and human pre- and post-implantation embryos and the stem cell lines that can be derived from them. Extra-embryonic tissues are shown in different shades of green, and epiblast derivatives in different shades of red. EPI: epiblast, TE: trophectoderm, PE: primitive endoderm (mouse), HYPO: hypoblast (human), ExE: extra-embryonic ectoderm, VE: visceral endoderm, AVE: anterior visceral endoderm, CT: cytotrophoblast, SCT: syncytiotrophoblast, YSE: yolk sac endoderm.

Figure 2: Images of stem cell embryo models. Oct4 labels pluripotent epiblast, cells, Brachyury marks mesoderm, Gata6 marks endoderm, Gata3 marks extra-embryonic cells, Sox2 labels both ectoderm and pluripotent cells, 7xTCF-mCherry is a reporter of Wnt signaling activity, E-Cadherin labels cell-cell adhesion sites, and Dapi and Hoechst label nuclei ESCs: Embryonic stem cells, TSCs: Trophoblast stem cells. Scale bars, 100 μm. These models are described in references (*34, 50, 52, 53, 56, 58, 59, 61*).

Figure 3: Summary of stem cell models of the mouse and human embryo. For each model the starting cell types, the corresponding embryonic stage, and the main advantages and disadvantages are shown. AVE: Anterior Visceral Endoderm, EMT: epithelial-to-mesenchymal transition, PASE: Post-implantation amniotic sac embryoid, ESCs: embryonic stem cells, TSCs: trophoblast stem cells, XEN cells: extra-embryonic endoderm stem cells.

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Figure 1

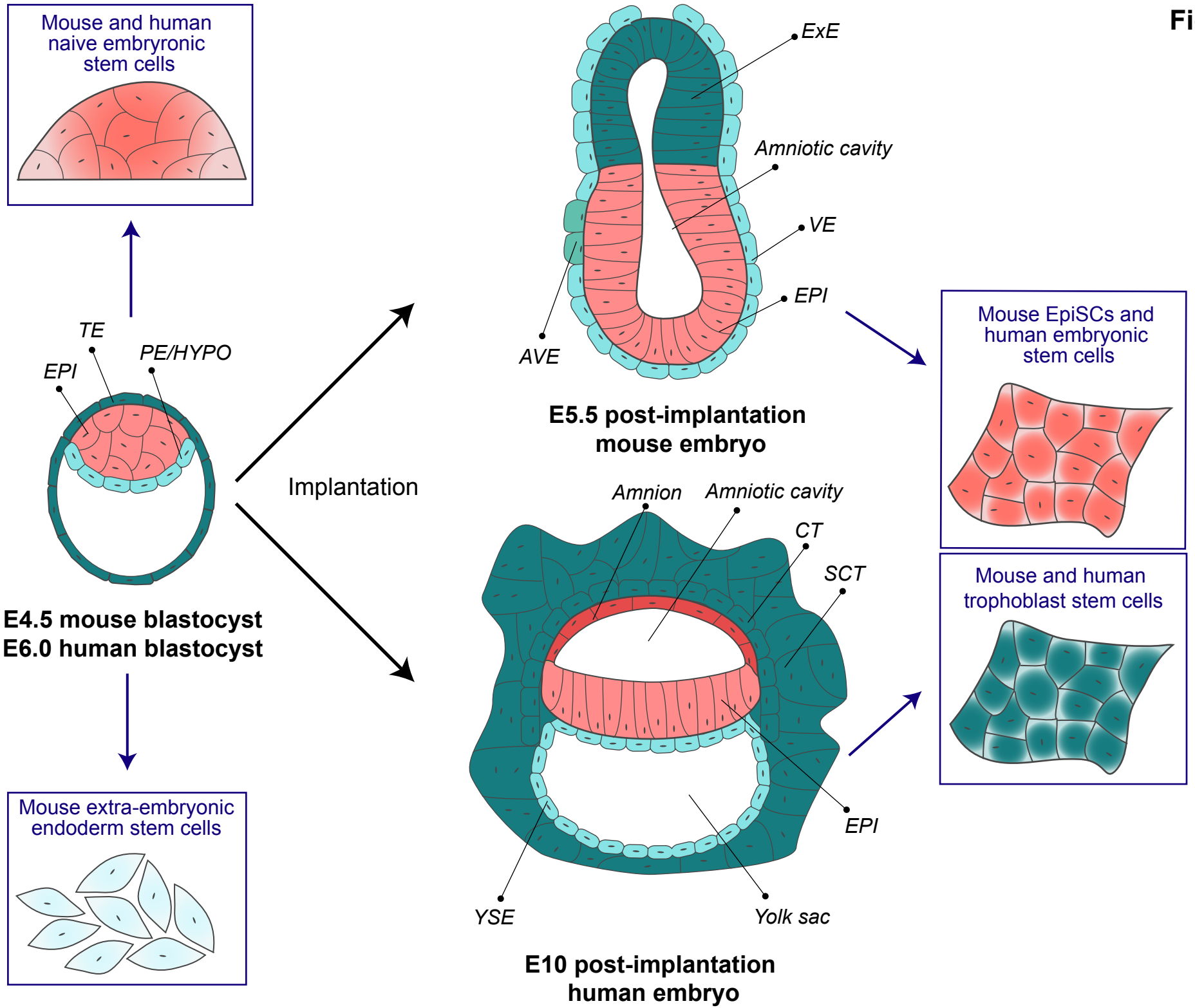
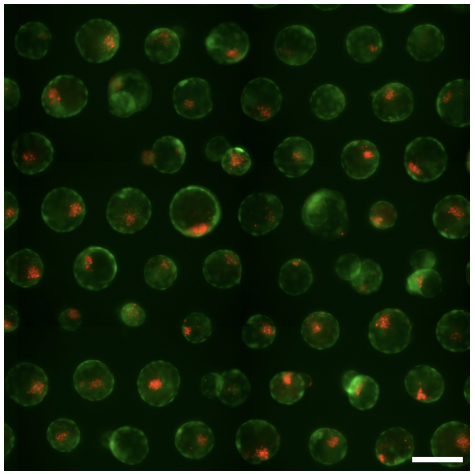

Mouse and human naive embryonic stem cells
TE
EPI
PE/HYPO
E4.5 mouse blastocyst
E6.0 human blastocyst
Implantation
Mouse extra-embryonic endoderm stem cells
ExE
Amniotic cavity
VE
EPI
AVE
E5.5 post-implantation mouse embryo
Mouse EpiSCs and human embryonic stem cells
Amnion
Amniotic cavity
CT
SCT
EPI
YSE
Yolk sac
E10 post-implantation human embryo
Mouse and human trophoblast stem cells

Figure 2

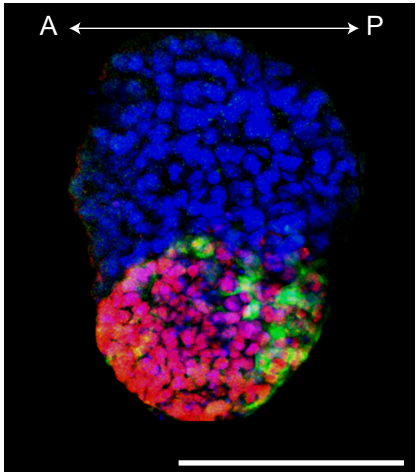
MOUSE

Blastoid



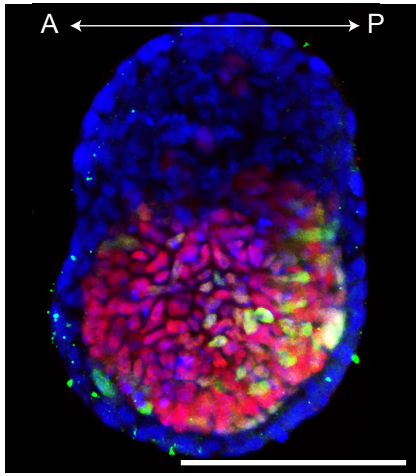
ESCs/TSCs

Polarized embryo-like structure



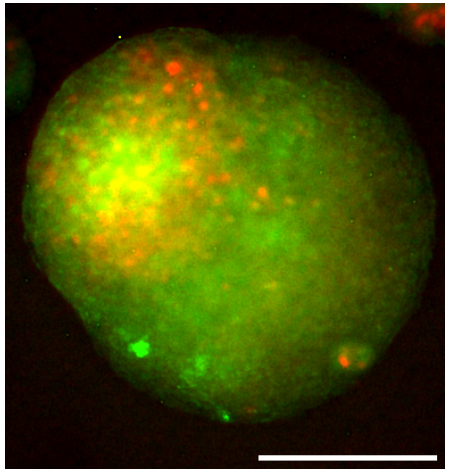
Oct4/Brachyury/Dapi

Gastrulating embryo-like structure



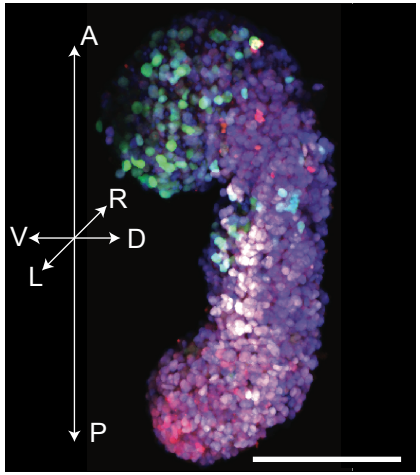
Oct4/Brachyury/Dapi

Polarized EB



Brachyury-GFP/7xTCF-mCherry

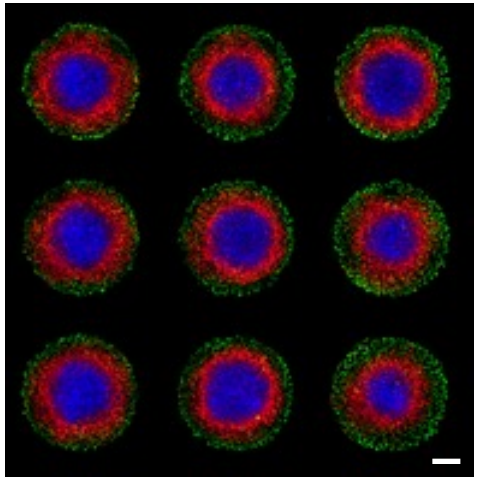
Gastruloid



Brachyury/Gata6::YFP /Hoechst/Sox2

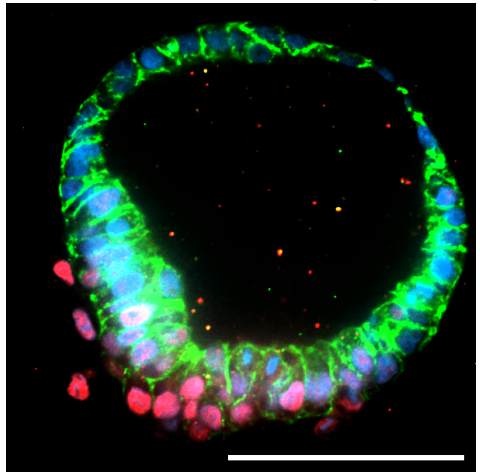
HUMAN

Micropatterned colonies



BRACHYURY/GATA3/SOX2

Post-implantation amniotic sac embryoid

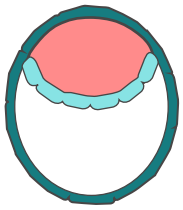
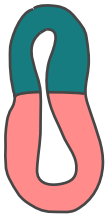
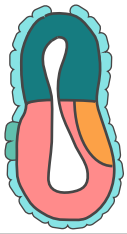
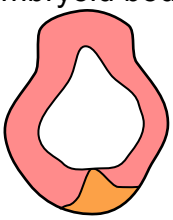
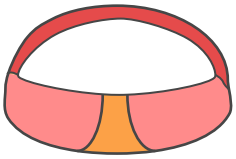
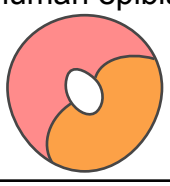


BRACHYURY/E-CADHERIN/DAPI

Asymmetric human epiblast

SOX2/BRACHYURY

Figure 3

EMBRYO-LIKE STRUCTURE	STARTING COMPONENTS	SPECIES	EMBRYONIC STAGE	ADVANTAGES	DISADVANTAGES
Blastoid 	ESCs + TSCs	Mouse	Blastocyst	- Self-assembly and self-organization between 2 cell types - Morphogenesis + cell fate	- Cellular interactions by chance (decreased efficiency) - Limited developmental potential
Polarized embryo like structure 	ESCs + TSCs	Mouse	Early post-implantation	- Self-assembly and self-organization between 2 cell types - Symmetry breaking in the absence of AVE	- Cellular interactions by chance (decreased efficiency) - Lack of EMT and gastrulation
Gastrulating embryo like structure 	ESCs + TSCs + XEN cells	Mouse	Gastrulation	- Self-assembly and self-organization between 3 cell types - Morphogenesis + cell fate	- Cellular interactions by chance (decreased efficiency) - Limited epiblast patterning
Embryoid body 	ESCs	Mouse	Post-gastrulation	- Self-organization of ESCs - Symmetry breaking in the absence of exogenous cues	- Limited patterning - Lack of proper tissue organization
Gastruloid 	ESCs	Mouse	Post-gastrulation	- Self-organization of ESCs - Cell fate specification and tissue patterning	- Limited morphogenesis - Lack of proper tissue organization
Micropatterned colonies 	ESCs	Human	Post-gastrulation	- Self-organization of ESCs - Quantitative cell fate specification and tissue patterning	- Limited morphogenesis - 2D platform
PASE 	ESCs	Human	Gastrulation	- Self-organization of ESCs - Spontaneous amnion-epiblast fate split	- Lack of precise control (decreased efficiency) - Limited epiblast patterning
Asymmetric human epiblast 	ESCs	Human	Early post-implantation	- Self-organization of ESCs - Spontaneous symmetry breaking	- Limited morphogenesis - Lack of morphological *in vivo* equivalent